Spring 5-1-2015

Physiological response to salinity challenge is mediated by Na+, K+ - ATPase "isoform switching" in a euryhaline fish, the Alewife

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ABSTRACT

Na^+, K^+-ATPase (NKA) α1a and α1b expression was investigated in Alewives in response to salinity challenge. Anadromous and landlocked Alewives were exposed to freshwater (0ppt) and seawater (30ppt) treatments and sampled at 2-, 5-, and 15-day time points. Anadromous alewives exhibit significant up-regulation of α1a in freshwater compared to seawater at all time points, and significant up-regulation of α1b in seawater compared to freshwater at all time points. In landlocked Alewives, α1a is significantly up regulated at the 5-day time point and α1b is significantly up regulated at all time points (in their respective salinities). Analysis of the ratio of α1b to α1a expression reveals a significant effect of salinity in both populations, and additionally a salinity by day interaction in the anadromous population. This significant expression difference between the isoforms denotes isoform switching in Alewives. Molecular phylogenetics performed using Geneious R8 with the Mr. Bayes plug-in show a single divergence evolution of the NKA isoforms in salmonids, but separate origins for the Alewife isoforms that may predate evolution in salmonids. Results of this study indicate that NKA α1a and α1b are present in Alewives and exhibit isoform switching in both anadromous and landlocked populations. Also, evidence shows that the evolutionary history of the isoforms may differ between taxa. Further studies should be done to investigate the presence of these isoforms in other species and create a more encompassing molecular phylogeny.

INTRODUCTION

Euryhalinity, the ability for fish to tolerate a wide range of salinity, has arisen independently many times in evolutionary history (Schultz and McCormick 2013). Euryhaline bony fish must be able to maintain ion and water homeostasis (a capacity known as osmoregulation) in both freshwater and seawater. This is particularly challenging given that these environments differ dramatically in salt concentration (10 mOsm vs. 1,050 mOsm: freshwater and seawater, respectively). Such osmoregulatory flexibility requires that fish shift between absorbing ions in a dilute freshwater environment and secreting ions in a concentrated seawater environment, a function that is achieved by a suite of ion pumps, channels, and transporters in specialized gill cells known as ionocytes (Evans et al. 2005).

Na^+, K^+-ATPase (NKA) is a key gill ion transport protein by which euryhaline fish are able to osmoregulate in different salinity environments. Two distinct isoforms of the NKA α subunit (the main catalytic subunit) may be particularly important to euryhalinity; NKA α1a and NKA α1b. The term "isoform" in this study is used in a general sense to describe different forms of the NKA alpha subunit. NKA α1a and α1b may be splice variants or paralogs, though recent evidence has identified them as paralogs (Dalziel et al. 2014). Freshwater or seawater acclimation induces differential expression of either NKA α1a (which predominates in freshwater acclimated fish) or NKA α1b (which predominates in seawater acclimated fish) in the gills of several euryhaline species (Urbina et al. 2012). Such salinity-dependent expression of NKA is an example of "isoform switching" and may be an adaptation to promote euryhalinity, though very few euryhaline taxa have been studied to date. This study attempts to

identify isoform switching in a previously untested euryhaline species, determine whether it is has arisen independently in different lineages via convergent evolution, and whether it is lost in populations that become specialized in freshwater.

NKA isoform switching was first discovered in Rainbow Trout (*Oncorhynchus mykiss;* Salmonidae) by Richards et al. (2003). Richards et al. (2003) utilized quantitative Real-time PCR to measure mRNA levels of α1a and α1b, and found an increase in α1a mRNA in freshwater and an increase in α1b mRNA upon seawater transfer. Researchers have also identified isoform switching in Atlantic Salmon (*Salmo salar;* Salmonidae) using Western blot to track salinity-dependent abundance of the protein product (McCormick et al. 2009). Three amino acid substitutions were identified that are thought to give NKA α1a and α1b isoforms distinctive electrochemical properties beneficial in freshwater and seawater, respectively. In particular, in αla these substitutions promote binding of sodium ions in the cytoplasm to reduce the work needed to actively transport sodium ions across the steep gradient from a dilute environment to the blood (Jorgensen et al. 2008). Such energetic efficiency might suggest why isoform switching may be an adaptive mechanism that facilitates euryhalinity.

Although isoform switching has been identified in several unrelated taxa, little is known about how this adaptive mechanism evolved (Salmonidae: Richards et al. 2003, Bystriansky et al. 2006, Jorgensen et al. 2008, and McCormick et al. 2009; Cichlidae: Tipsmark et al. 2011; Galaxiidae: Urbina et al. 2012). Two alternative hypotheses for its evolutionary origin are that it: (1) evolved independently in multiple, unrelated lineages (convergence); or, (2) evolved once in an ancestral taxon deep in the evolutionary history of fishes (single divergence). Past molecular evidence supports the first hypothesis:

Urbina et al (2012) produced a molecular phylogenetic tree that depicts an absence of separate α1a and α1b groupings, wherein α1a and α1b are more closely related to each other within a species than to their counterparts in different species. For example, α1a in Atlantic Salmon is more closely related to α1b in the Atlantic Salmon then it is to an α1a in any other species. This type of relationship between isoforms provides evidence for the hypothesis of independent or convergent evolution (Urbina et al 2012). Convergent evolution of these isoforms is especially interesting because they have all or most of the same three amino acid substitutions mentioned previously. It is possible that selection is strong enough on the isoforms to drive them to evolve the same substitutions. More recent evidence found by Dalziel et al. (2014) suggests that NKA α1a and α1b were derived in a common ancestor. The molecular phylogeny built by Dalziel et al. (2014) displays clades that are isoform specific, but only including Salmonidae. A gene duplication event in Salmonidae may be the point of origin for NKA α1a and α1b in this family. In this case, members of the Salmonidae have retained the isoforms evolved from the duplication event instead of deriving them independently (Dalziel et al. 2014). The opposing evidence found by Urbina et al. (2012) and Dalziel et al. (2014) concerning the evolution of NKA isoforms is an important finding and requires further corroboration with the study of additional species.

In this study, the Alewife (*Alosa pseudoharengus*), a euryhaline teleost in the family Clupeidae, is used to explore the evolutionary origins of NKA isoform switching. I predict that NKA isoforms in the Alewife gill will exhibit isoform switching and that freshwater and seawater NKA isoforms evolved independently. To test this, I attempt to identify candidate isoforms in Alewife using the isoform-specific amino acid

substitutions found by Jorgensen et al. (2008), and verify the presence of isoform switching through quantitative Real-time PCR by testing for salinity-dependent expression of NKA αla and α1b. Candidate Alewife α1a and α1b isoforms are placed on a molecular phylogenetic tree with all other α1a and α1b isoforms known for fish using data from Urbina et al. (2012). If the prediction for convergent evolution is correct, the α1a and α1b isoforms will group within a species (similar to what Urbina et al. (2012) found). Alternatively, findings could suggest that isoform switching evolved once in a deep teleostean ancestor (as suggested by Dalziel et al. 2014). If this single divergence is the case, my molecular phylogenetic tree will form an α1a clade separate from an α1b clade, effectively grouping by isoform rather than by species. This would indicate that the α1a and α1b isoforms are more related to their counterparts in other species than to one another within species. In this case, α1a may have evolved in an early ancestor and persisted in emergent species, and α1b may have evolved separately in the same ancestor or in a later ancestor. Evolution in a common ancestor may be a likely solution for the origin of the α1a and α1b isoforms, but it is possible that evolutionary history may vary between taxa.

Evidence for differentiation of NKA isoform switching in life history forms may be found by investigating changes to salinity-dependent isoform expression in a landlocked (i.e., freshwater-restricted) population of a euryhaline species in which seawater has been eliminated from the life cycle. For this reason, Alewives present an exceptional opportunity to investigate the adaptive importance of NKA isoform switching, as independently evolved landlocked populations of Alewives exist in Connecticut and were formed 300 to 5,000 years (Palkovacs et al. 2008). A previous

study shows that landlocked Alewives have reduced tolerance of seawater and slightly enhanced tolerance of freshwater relative to anadromous Alewives (Velotta et al, 2014). I plan to use gill tissue sampled from freshwater and seawater challenged landlocked and anadromous Alewives to investigate possible expression differences between these populations. I expect to find that expression of α1a in freshwater will be higher and expression of α1b in seawater will be lower among landlocked compared to anadromous Alewives. The reduction of reciprocal α1a and α1b expression in the landlocked population is feasible because of their specialization to freshwater and the association of freshwater tolerance to α1a expression. This result might signify the adaptive nature of these isoforms by indicating a correlation between the magnitude of isoform switching and the presence of life history traits involving different salinity environments This might also indicate that in the anadromous population anadromy and isoform switching are adaptively linked. An alternative prediction would be that the landlocked Alewives would display the same capacity for isoform switching as the anadromous Alewives. This would suggest that the reduction in seawater tolerance in the landlocked population is a result of changes to other osmoregulatory mechanisms.

MATERIALS AND METHODS

Sample Collection and Treatment

Samples are used from a previous study (Velotta et al. 2014) in which young of the year Alewives (*Alosa pseudoharengus*) were captured via purse seine after dusk using a floating pool light as an attractant. Bride Lake in East Lyme, Connecticut was sampled for anadromous alewives, and Rogers Lake in Old Lyme, Connecticut was sampled for

landlocked Alewives in October 2011. Captured fish were brought to the Conte Anadromous Fish Research Lab in Turners Falls, Massachusetts and kept at 0.5ppt salinity before undergoing treatments. Captive alewives were then subjected to 2, 5, or 15 days of either 0ppt or 30ppt salinity treatment in 1,200-liter tanks equipped with charcoal filters. Once treatments were completed, Alewives were euthanized with MS-222 and gill tissue was obtained. RNA was later extracted from homogenized gill tissue using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), DNase treated using the TURBO DNA-free kit (Life Technologies, Grand Island, NY, USA), and stored at -80. I reversed transcribed samples using about 500 ng of RNA with the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies, Grand Island, NY, USA) to obtain cDNA for quantitative analysis.

Choosing and Testing Primers

Candidate sequences were identified using NCBI BLAST and an existing Alewife transcriptome from Velotta et al, (2014). I built nucleotide alignments containing select candidate sequences and known NKA α1a and α1b sequences from previous studies (Richards et al. 2003, Bystriansky et al. 2006, Jorgensen et al. 2008, McCormick et al. 2009, Tipsmark et al. 2011, and Urbina et al. 2012) using Geneious and CLC Genomics. Transcripts were filtered by analyzing alignments for Alewife sequences most closely matched to the previously identified sequences, and with the fewest gaps. I designed sequence specific primers for chosen candidates, and then tested them using PCR and gel electrophoresis.

I chose two products that I believed to be α1a and α1b, and brought them to the University of Connecticut DNA Biotechnology Facility for sequencing. Sequences were

aligned with previously identified NKA α1a and α1b sequences in different species of fish. Alignments were performed with Geneious and CLC. Conserved regions between these sequences were identified and then assessed based on mismatches and gaps to determine the likelihood that the candidates were viable. Functionally important protein substitutions found by Jorgensen et al. (2008) were considered, but Alewife NKA α1a contained none of the regions containing substitutions and α1b contained only one (fig. 1).

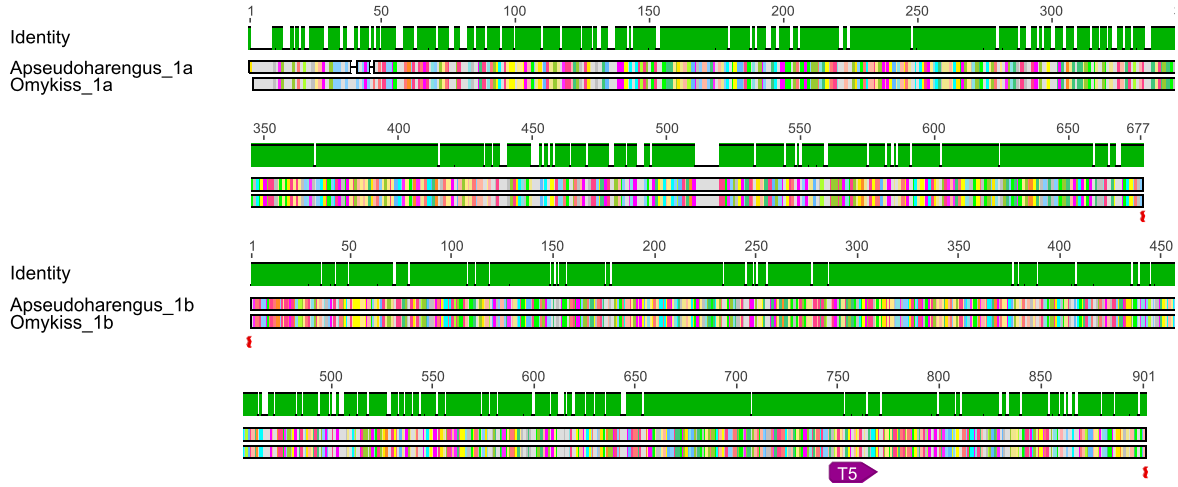

Figure 1: Protein alignment of anadromous Alewife NKA α1a and α1b with Rainbow Trout sequences. Proteins are color-coded, so mismatched colors indicate mismatched amino acid sequence. The green bar indicates sequence agreement. Gaps in the green bar signify sequence disagreements. The purple annotation labeled T5 represents transmembrane region 5 of Rainbow Trout where Jorgensen et al. (2008) identified a functionally important amino acid substitution. The region only has 4 mismatches between Rainbow Trout and Alewife, but the single amino acid substitution that was deemed functional is not present in the Alewife sequence (it is one of the mismatches).

New primers compatible with quantitative analysis were made for the resulting sequences (Table 2) to test for isoform switching and confirm their identity as NKA α1a and α1b. The suspected NKA α1a was confirmed to show salinity-dependent expression

with gel electrophoresis, but the NKA α1b candidate failed. A second attempt at primer design yielded an NKA α1b candidate that exhibited the expected salinity-dependent expression.

Table 2 - qPCR Primers

Name	Sequences	Primer Success
13_F	GGTGGCAGAGGAGCAGTC	Y – confirmed α1a
13_R	CGACGCACAGATCCACAG	Y – confirmed α1a
14.1_F	AGACAGGAACCGCTTTTGAC	Y – confirmed α1b
14.1_R	TCTTGAGGATGGGGACATTC	Y – confirmed α1b

Real-time Quantitative PCR

Real-time quantitative PCR (qPCR) was performed to analyze expression of the NKA α1a and α1b isoforms. Six samples from each treatment group were used in this assay as RNA samples were limiting. I used a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA, USA) to run qPCR with Bio-Rad's iTaq Universal SYBR Green Supermix. I ran Samples in triplicate on 96-well plates. Conditions of the thermocycler were as follows: 10 minutes at 95°C, 45 cycles of 95°C for 20 seconds and 60°C for 50 seconds. Primer efficiencies were calculated as E-values before data was deemed reliable and all were found to be acceptable. The ideal E-value is 2, and all primer pair efficiencies approached a value of 2 (EF1a = 1.9, 13 (α1a) = 2.1, and 14.1 (α1b) = 2.0) The E-value is calculated as:

$$E = 10^{-\frac{1}{S_d}}$$

where s_d is the slope of the line created by plotting the Cq of the sample for three dilutions (1:1, 1:10, 1:100) against the magnitude of dilution (0, -1, -2). All samples were

run in triplicate, and dilution series were run three times and averaged to obtain the E-value.

Data and Statistics

I used EF1α as a reference gene in accordance with a previous study (Velotta et al, 2014). Reference genes should show no changes in expression under experimental conditions, and are used in case cDNA yield from reverse transcription varies among samples. To account for differences in cDNA yield of experimental samples, relative expression is normalized using the following equation:

$$\Delta\Delta C_T = \frac{E_{tar}^{\Delta C_T tar(calibrator-test)}}{E_{ref}^{\Delta C_T ref(calibrator-test)}}$$

Data was also normalized by calibrator samples because of significant plate-to-plate variability for EF1α (One way ANOVA: $F_{11,24} = 50$, $p = 0.001$). There was also a strongly correlated plate effect for EF1α and both isoforms (NKA α1a: $r = 0.94$, $p = 0.006$; NKA α1b: $r = 0.97$, $p = 0.001$). Since the qPCR assays were done using multiple 96-well plates, it is possible that one plate exhibited higher overall expression than another plate due to random effects or error. Normalizing data with the calibrator sample (relativizing experimental sample expression to calibrator sample expression) accounts for random plate differences. All data used for statistical analyses of gene expression is log transformed so that there is no variance and mean relationship ($r = -0.34$, $p = 0.07$).

Gene expression of EF1α was investigated under experimental conditions, and in non-normalized data an effect of salinity and life history form was present. Salinity responses of EF1α were notable lesser than NKA α1a, so results may not be significantly skewed. Also, it is possible that there is no salinity response, and changes in expression

are simply due to differences in cDNA yield. To eliminate bias from EF1α, a measure of gene expression was used that does not factor in a reference gene.

Gene expression was also investigated as a ratio of α1b to α1a expression relativized to the calibrator samples, here on referred to as the "ratio-ratio". The ratio-ratio of the gene expression relativizes gene expression using the two experimental genes instead of a reference gene to investigate expression differences.. This removes error that may be incurred by the use of the EF1α reference gene.

All statistical analyses were performed using SAS. Mixed model analysis was used so that models would include both fixed effects and random effects. The effect of tank was coded as a random effect in the models. Experimental treatments had two tanks each in case of contamination or other mortality inducing events. Random differences between tanks may exist, and the mixed model analysis accounts for this possibility. Full models had length, salinity, length x salinity, day, length x day, salinity x day, and length x salinity x day variables ("variable x variable" indicates an interaction variable).. Models were reduced by removing statistically insignificant variables while considering AIC, AICC, and BIC. These scores use data fit and model complexity to provide information about which model best fits the data. The final model for all mixed model analyses included length and salinity as variables.

Phylogenetic Analysis

Previously identified sequences obtained from NCBI GeneBank or Ensembl (table 2) were compiled into Geneious R8 software. I identified Alewife sequences for NKA α1a and α1b by realigning sequenced products with the parent sequence originally

used to design primers (table 3). I made nucleotide alignments using the Geneious align feature. The alignment was then used for Bayesian phylogenetic analysis using the Mr. Bayes plugin for Geneious R8.

Table 2: Previously identified sequences used, in this study, for molecular phylogenetics.

Species	Genbank Accession number or Ensembl ID	Name in Phylogeny
Salmo salar	BT058747.1	Ssalar_1b
Oncorhynchus mykiss	NM_001124461.1	Omykiss_1a
Oncorhynchus mykiss	NM_001124460.1	Omykiss_1b
Oncorhynchus mykiss	NM_001124459.1	Omykiss_1c
Oncorhynchus mykiss	NM_001124458.1	Omykiss_2
Oncorhynchus mykiss	NM_001124630.1	Omykiss_3
Oncorhynchus masou	AB573640.1	Omasou_1a
Oncorhynchus masou	AB573639.1	Omasou_1b
Oreochromis niloticus	ENSONIT00000015703	Oniloticus_1-1
Oreochromis niloticus	ENSONIT00000015672	Oniloticus_1-2
Oreochromis niloticus	ENSONIT00000015628	Oniloticus_1-3
Oreochromis niloticus	ENSONIT00000015603	Oniloticus_1-4
Anabas testudineus	JN180940	Atestudineus_1a
Anabas testudineus	JN180941	Atestudineus_1b
Anabas testudineus	JN180942	Atestudineus_1c
Takifugu rubripes	ENSTRUT00000032672	Trubripes_1-1
Takifugu rubripes	ENSTRUT00000033934	Trubripes_1-2
Tetraodon nigroviridis	ENSTNIT00000009334	Tnigroviridis_1-2
Tetraodon nigroviridis	ENSTNIT00000009181	Tnigroviridis_1-2
Gasterosteus aculeatus	ENSGACT00000018945	Gaculeatus_1-1
Gasterosteus aculeatus	ENSGACT00000018961	Gaculeatus_1-2
Salvelinus alpinus	KJ175154	Salpinus_1a
Salvelinus alpinus	KJ175155	Salpinus_1b
Salmo salar	KJ175156	Ssalar_1a
Salmo salar	KJ175157	Ssalar_1c
Thymallus arcticus	KJ175158	Tarcticus_1a
Thymallus arcticus	KJ175159	Tarcticus_1b
Coregonus clupeaformis	KJ175160	Cclupeaformis_1a
Coregonus clupeaformis	KJ175161	Cclupeaformis_1b
Esox lucius	KJ175162	Elucius_1a-x
Esox lucius	KJ175163	Elucius_1a-y
Esox lucius	KJ175164	Elucius_1b
Esox lucius	KJ175165	Elucius_1c
Osmerus mordax	KJ175166	Omordax_1-1
Osmerus mordax	KJ175167	Omordax_1-2

Table 3: Anadromous Alewife NKA α1a and α1b sequences. The α1a sequence is in frame for translation, but the α1b sequence needs translation frame 2 for accurate protein sequence.

Isoform	Nucleotide Sequence
α1a	TGTTCATCCAGAAGAGAAGTACCGACCGCCACCATGGGATACGGGGCCG GAAGAGACAAGTATGAGCCTGCAGCTACCTCTGAGCAGGGGGGCAAGAA GAAAAAGGGAAAGGGAAAGGGGAAAGAAAAAGACATGGACGAGCTAAA GAAGGAAGTGACCATGGAGGACCACAAACTGAGCCTGGATGAGCTGCAC CGCAAGTTTGGGACAGACCTGCAAAAAGGTCTGACCACAGCCCGGGCAG CAGAGATCCTGGCACGAGATGGACCCAATGCCCTCACCCCACCCCCTACT ACTCCAGAGTGGGTGAAATTTTGCCGCCAGCTGTTTGGGGGTTTCTCTAT GTTGCTGTGGATTGGAGCCATACTCTGCTTCCTGGCCTATGGTATCCAAG CTGCCATGGAAGAGGAGCCACAGAATGATAATCTCTACCTCGGTGTTGTC TTGTCTGCTGTCGTTATCATCACTGGCTGCTTCTCCTACTATCAAGAGGCC AAAAGCTCAAAGATCATGGAGTCCTTCAAGAACATGGTTCCTCAGCAAG CCCTGGTAATCCGAAGCGGAGAGAAGCTGAGTATCAATGCAGAAGAAGT GGTTTTGGGTGACCTGGTAGAAGTGAAGGGAGGAGATCGGATACCTGCG GATCTTAGGGTCATCTCCTCTCATGGCTGCAAGGTGGATAACTCTTCCCTC ACTGGTGAGTCCGAGCCCCAGACCCGCTCTCCTGACTTCACCAATGAGAA CCCGCTGGAGACTCGTAACATTGCTTTCTTCTCTACAAACTGTGTGGAAG GCACAGCCCGTGGAATTGTCGTGAACACCGGCGACCGTACAGTGATGGG TCGTATTGCCACTCTGGCTTCTGGTCTGGAGGGAGGACGCACCCCCATTG CAATTGAAATTGAGCATTTTATCCACATCATCACAGGCGTGGCAGTCTTC CTGGGAGTCTCCTTCTTCGTCCTGTCACTCATTCTGCAGTACACTTGGCTG GAGGCTGTCATCTTCCTCATTGGGATCATTGTTGCCAACGTGCCAGAGGG GCTGCTGGCTACTGTCACGGTGTGCCTCACACTGACCGCCAAAAGAATGG CCCGCAAGAATTGTCTTGTAAAGAATCTGGAAGCTGTGGAGACATTGGG ATCCACTTCTACCATTTGCTCTGACAAAACTGGGACCTTAACGCAGAACC GAATGACTGTAGCCCATATGTGGTTTGATAACCAGATCCATGAGGCTGAC ACTACCGAGGACCAGTCAGGAACCTCGTTTGACAAGAGCTCGCACACCT GGGTGGCCCTGTCCCACATTGCCGGACTCTGCAACCGTGCCGTCTTCAAG GGAGGTCAGGACAACATCCCGGTGCTCAAGAGGGATGTGGCCGGGGATG CCTCTGAGTCTGCCCTGCTCAAGTGCATCGAGCTGTCCTCTGGCTCCGTGA AGCTGATGCGTGAACGTAACAAGAAAGTGGCCGAGATTCCCTTCAATTCC ACCAACAAATACCAGCTCTCCATCCACGAGACCGAAGACCCCAATGACA ACCGATACCTGCTAGTGATGAAGGGGGCCCCCGAGCGCATCCTGGACCG TTGCTCCACCATCCTGCTGCAGGGCAAGGAGCAGCCGCTGGATGAGGAG ATGAAGGAGGCCTTCCAGAACGCCTACCTGGAGCTGGGCGGTCTGGGTG AAAGAGTGCTCGGCTTCTGCCATTTCAACCTGCCTGATGACCAGTTCTCT GAGGATTTCGTCTTTGACTGTGAGGAGGTGAACTTCCCCACCGAGAATCT GTGCTTCATTGGCCTCATGTCCATGATTGACCCTCCTCGTGCTGCTGTGCC CGATGCTGTTGGCAAGTGCAGGAGTGCTGGAATCAAGGTCATCATGGTG ACTGGTGATCATCCCATCACTGCTAAGGCCATCGCCAAAGGTGTAGGCAT CATCTCTGAGGGTAACGAGACCGTGGAAGATATTGCAGCTCGCCTGAAC ATTCCCATTACAGAAGTCAACCCAAGAGATGCCAAGGC
α1b	AAATATGGATGACCTGAAGAAAGAAGTAGATCTGGATGACCACAAACTG ACCTTGGATGAGCTTCACCGCAAATATGGAACAGACCTGCAGAGGGGTC TGACCTCCACTCGTGCAAAAGAGGTCCTTGATCGTGATGGTCCCAATTCT CTGACCCCACCACCCACCACCCCAGAATGGGTGAAGTTCTGCAAGCAGCT CTTTGGTGGGTTCTCCACTCTGCTGTGGATTGGAGCCATCCTCTGCTTCCT

	GGCTTACGGTATTCAGGCTGCCTCAGAAGATGAACCAGCAAATGATAATC TGTACTTGGGCGTTGTGCTATCTGCTGTCGTCATCATCACTGGCTGTTTCT CCTACTTTCAAGAAGCCAAGAGTTCAAAGATTATGGAGTCCTTTAAGAAC CTGGTCCCTCAGCAAGCTCTGGTTGTGCGTGACGGAGAAAAAAAGAGCA TCGATGCTGTGGAGGTGGTGGCTGGAGATCTGGTGGAGGTGAAAGGCGG AGACAGAATCCCTGCTGACCTGCGTATCATCTCTGCTCATGGCTGCAAGG TGGACAACTCCTCCCTCACAGGAGAATCTGAGCCTCAGACACGTACCCCT GACTTCTCCAATGATAACCCCCTGGAAACTAGGAACATCGCTTTCTTCTC CACTAACTGTGTCGAAGGTACTGCTAGAGGTATTGTCATCAACACTGGTG ATCGCACAGTCATGGGTCGTATTGCCACCCTGGCTTCTGGCCTTGAAGTC GGCCGCACACCCATCTCCATTGAAATCGAGCACTTCATCCACATCATCAC TGGTGTGGCTGTCTTCCTGGGTGTCTCGTTCTTCATCCTCTCGCTCATCCTT GGATACAGCTGGCTGGAGGCGGTCATCTTCCTCATCGGGATCATTGTTGC CAACGTGCCGGAAGGTCTCCTGGCTACAGTCACTGTGTGTCTGACCTTGA CTGCCAAGCGCATGGCCAAGAAGAACTGCCTGGTGAAGAACCTGGAAGC TGTGGAGACTCTGGGCTCTACGTCCACCATCTGCTCTGACAAGACCGGAA CTCTGACCCAGAACCGCATGACGGTGGCCCACATGTGGTTTGACAACCAG ATCCATGAAGCCGACACCACTGAGAACCAGACAGGAACCGCTTTTGACC GCTCATCAGCAACCTGGAACTCCCTGGCACGCGTGGCTGGTCTGTGCAAC CGTGCTGTCTTCCTGGCAGATCAGCAGAATGTCCCCATCCTCAAGAGAGA CACAGCTGGTGACGCCTCTGAGTCTGCCCTGCTGAAGTGCATTGAGCTGT GCTGTGGCCCGGTGAAAGAGATGCGTGAGAAGTACCAGAAGCTTGCTGA GATCCCCTTCAACTCCACCAACAAGTACCAGCTCTCCATCCATGTCAATC CTGATACCTTGGAGTCAAAGCACCTGCTGGTCATGAAGGGAGCACCAGA GAAGATTCTAGAACGCTGTTCCACCATCCTCATCGAGGGCAAAGAGCAG CGTCTCGACGAAGAGATGAGGGCTGCGTTCAACAACGCCTATATGGAGC TGGGAGGCAGGGGAGAGAGAGTGCTGGGTTTCTGTCATCTCCCCCTTCCT GACGGGCAGTTTCCCGAGGGCTTTAAGTTTGACACGGACGAGATGAACTT CCCCACCGAGGGCCTGTGCTTCCTTGGCCTCATGTCCATGATCGACCCCC CGCGTGCTGCCGTGCCTGATGCCGTGCGCAAGTGCAGAACCGCTGGAATC AATGTCATCATGGTCACAGGTGACCACCCAATTACTGCAGGGGCCATAGC CAGGGCTGTGGGCATCATATCCGAGGGCAGTGAAACAGTGAAGGAAATG GCAGAACGACTGAACGTGCCTGTTAGCGAGATCAATCCAAGAGATGTGA AGGCCTGCGTGATCCACGGTGGGGAGCTGAAGGACATGACGTCCGAGCA GCTGGACGACATCCTGAGGAACCACACAGAGATTGTGTTTGCCAGAACCT CTCCGCAGCAGAAGCTCATCATCGTTGAAGGTTGTCAGCGGCAGGGTGCT ATTGTGGCAGTGACGGGCGACGGGGTCAATGACTCTCCAGCTCTGAAGA AGGCCGACATTGGCGTCGCCATGGGTATCGCTGGGTCTGACGTCTCCAAG CAGGCTGCTGACATGATCCTCTTGGATGATAACTTTGCCTCCATCGTCAC AGGAGTCGAAGAGGGGCGTTTGATCTTTGATAACTTGAAGAAGTCCATCG CTTACACGCTGACCAGTAAAATCCCTGAGATGTCACCGTTTCTCTTCTTCG TCATCGCTAACATCCCCCTGGCTCTTGGCACGGTCACCATCCTCTGTATCG ACCTGGGCACTGACATGGTTCCTGCCATCTCCCTGGCGTATGAGAAGGCG GAGAGCGACATCATGAAGAGACAGCCCAGAGACCCTGTGGCGGACAAGC TGGTCAACGAGAGGCTGATTAGCGTAGCCTACGGTCAGATTGGAATGATC CAAGCAGTGGGAGGATTCTTCACCTACCTTGTGATCCTGGCTGAGAATGG CTTCCTGCCCATGGACCTCTTTGGAATTCGAGTCTCCTGGGAAGACAAGT ACAACAACGAGCTCGAGGACAGTTATGGCCAGCAGTGGACATACGAGAG CAGAAAGATTGTGGAGTACACGTGCCACACAGCGTTTTTCGTGAGCATCG TCATCGTGCAGTGGAC

RESULTS

Presence of Isoform Switching

Isoform switching in Alewives has been identified. The candidate gene for NKA α1a shows significant up-regulation in freshwater and down-regulation in seawater as is expected (fig. 2). In the anadromous population there is a significant effect of salinity (ANOVA: $F_{1, 25} = 5.2$, $p = 0.03$), as well as a significant interaction of salinity and day (ANOVA: $F_{2, 25} = 42$, $p = 0.0001$). Effect of salinity on α1a expression is significant at all time points (table 4). Expression of NKA α1b is also significant at all time points (table 4). Isoform switching of NKA α1a and α1b is evident in the ratio-ratio graph (fig. 4), which has a significant salinity effect (ANOVA: $F_{1,25} = 5.22$, $p = 0.0311$) and salinity by day interaction (ANOVA: $F_{2,25} = 31.64$, $p = <.0001$).

Table 4: Statistical analysis of expression by day in anadromous Alewives

NKA α1a				
Day 2 - Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Lt	1	8	0.77	0.4065
salinity	1	8	54.18	<.0001
Day 5 - Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Lt	1	8	0.67	0.4375
salinity	1	8	1123.02	<.0001
Day 15 - Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Lt	1	8	0.28	0.6142
salinity	1	8	161.13	<.0001
NKA α1b				
Day 2 - Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Lt	1	8	0.22	0.6496
salinity	1	8	362.78	<.0001

Day 5 - Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Lt	1	8	0	0.9842
salinity	1	8	411.66	<.0001
Day 15 - Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Lt	1	8	0.04	0.8552
salinity	1	8	183.3	<.0001

Expression in Landlocked Alewives

Expression of the NKA isoforms in landlocked Alewives shows significant salinity effect of α1a at an intermediate time point and α1b at all time points. There is a significant effect of salinity at day 5 for NKA α1a (table 5). However, NKA α1a expression does not increase to the same extent in the landlocked population as in the anadromous population (fig. 5). A significant effect of salinity on expression of NKA α1b is present at all time points (fig. 6 , table 5). Isoform switching can also be seen in the ratio-ratio graph (fig. 7), which has a significant salinity by day interaction effect (ANOVA: $F_{2,25} = 5.67$, $p = 0.0093$).

Table 5: Statistical analysis of expression by day in landlocked Alewives

NKA α1a				
Day 2 - Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Lt	1	8	7.78	0.0236
salinity	1	8	4.83	0.0592
Day 5 - Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Lt	1	8	0.36	0.5672
salinity	1	8	27.04	0.0008
Day 15 - Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Lt	1	8	0.53	0.4884
salinity	1	8	4.65	0.0631

NKA α1b				
Day 2 - Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Lt	1	8	3.22	0.1103
salinity	1	8	39.05	0.0002
Day 5 - Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Lt	1	8	0.48	0.508
salinity	1	8	171.54	<.0001
Day 15 - Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Lt	1	8	0.62	0.4522
salinity	1	8	11.27	0.01

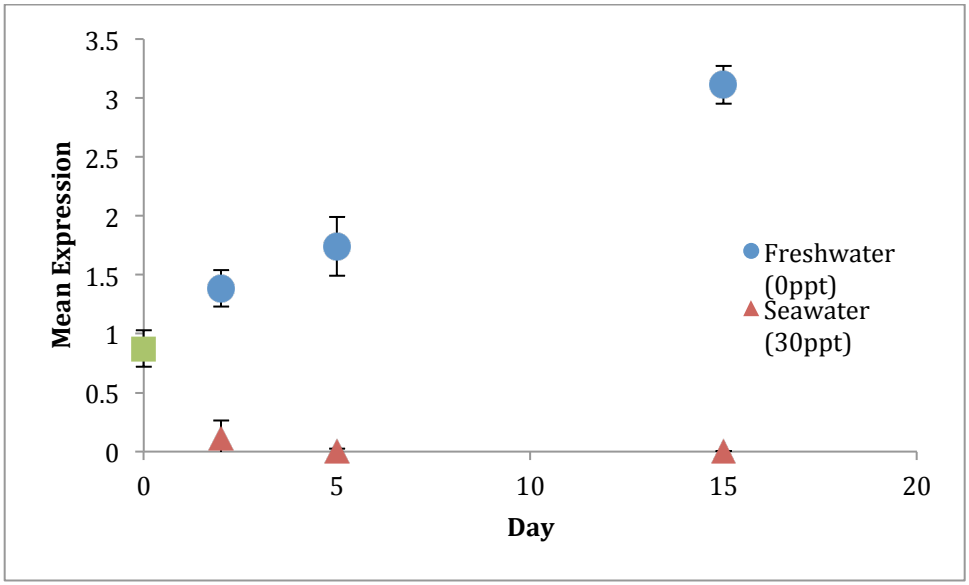

Figure 2: Mean expression of NKA α1a in anadromous Alewives during a multiday salinity challenge.

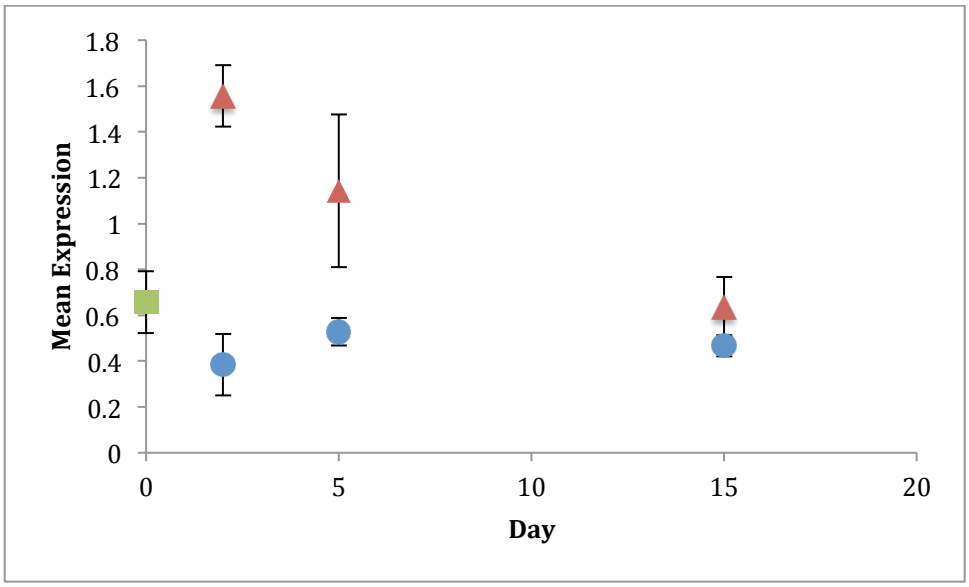

Figure 3: mean expression of NKA α1b in anadromous Alewives during a multiday salinity challenge.

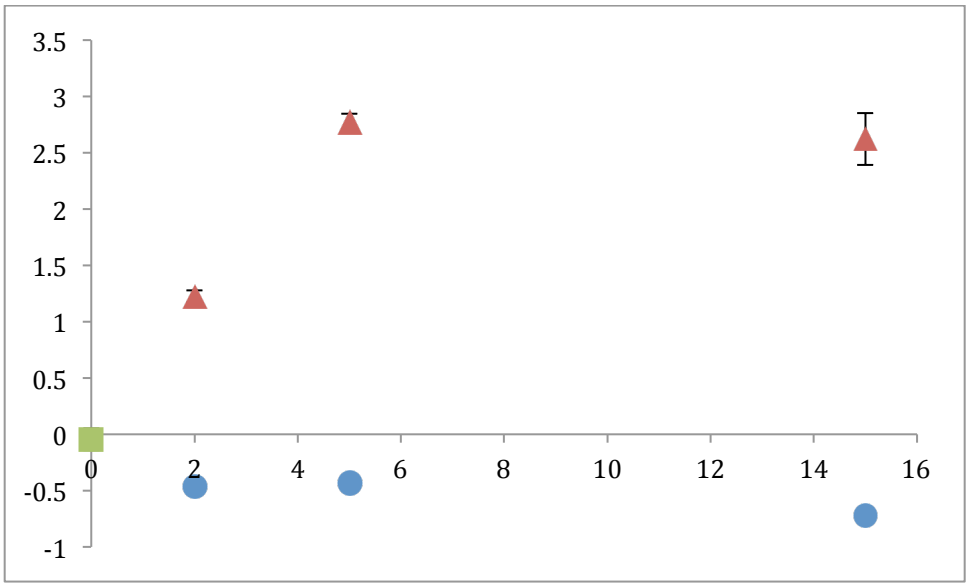

Figure 4: Ratio-ratio expression of NKA isoforms in anadromous Alewives in response to a multiday salinity challenge.

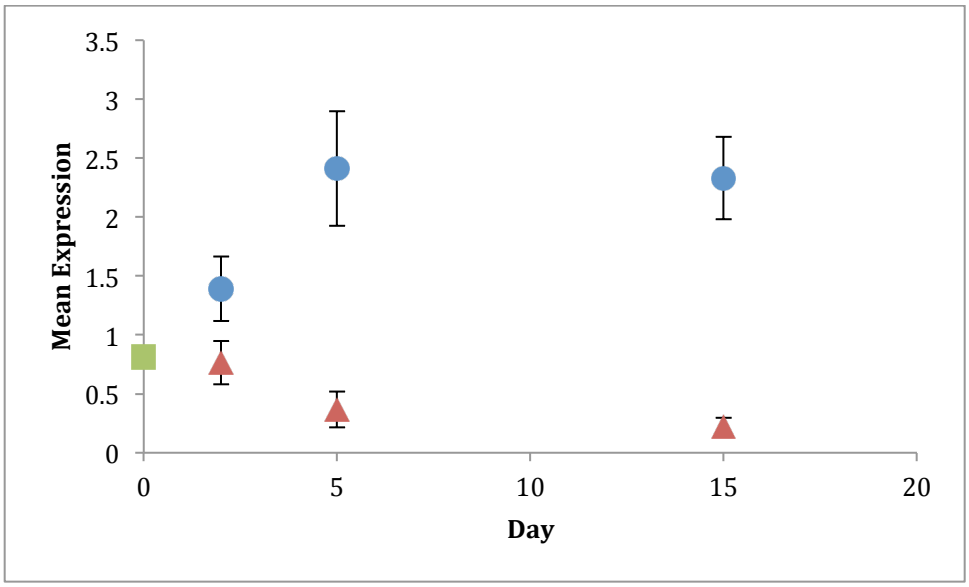

Figure 5: Mean expression of NKA α1a in landlocked Alewives during a multiday salinity challenge.

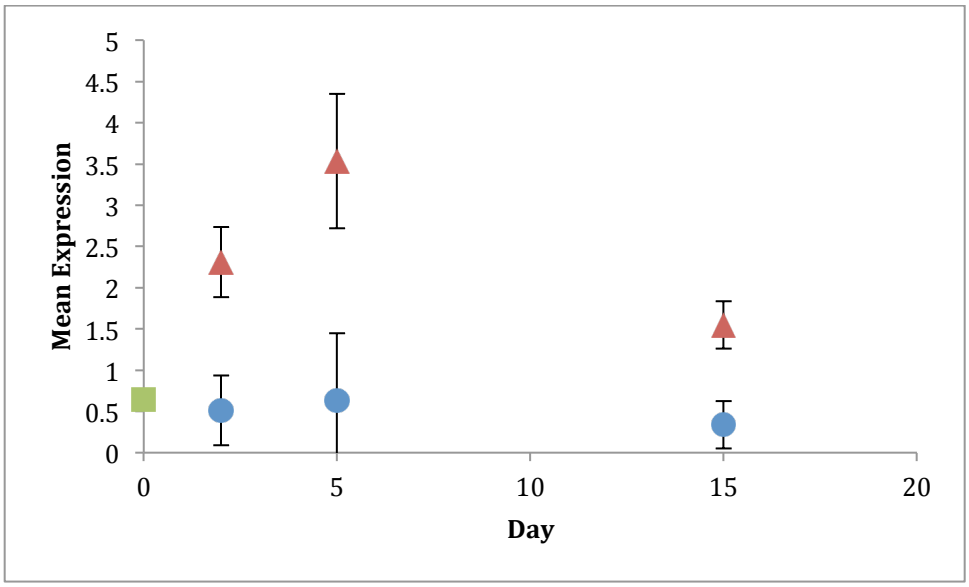

Figure 6: Mean expression of NKA α1b in landlocked Alewives during a multiday salinity challenge.

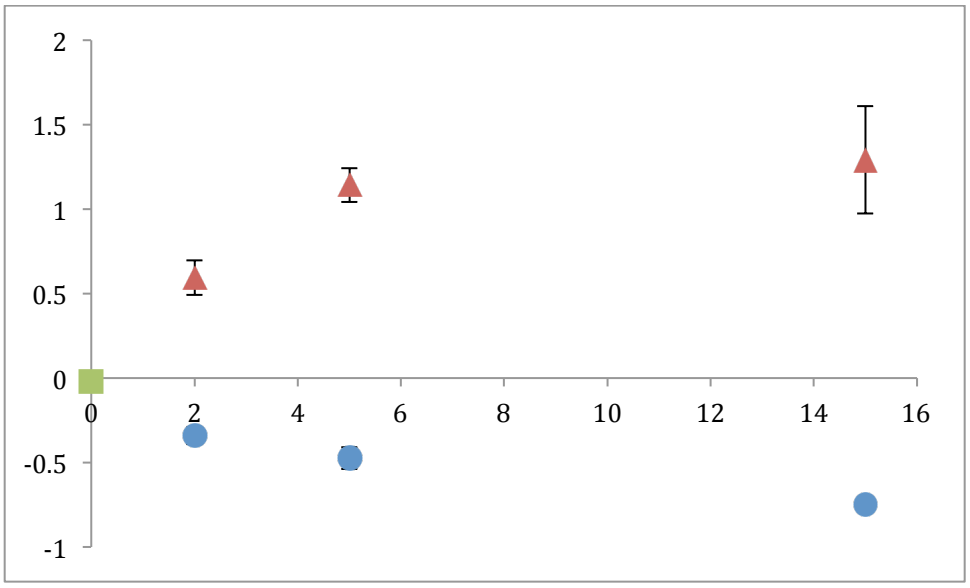

Figure 7: Ratio-ratio expression of NKA isoforms in landlocked Alewives in response to a multiday salinity challenge.

Molecular Phylogeny

The molecular phylogeny built in this study may be seen in figures 8 and 9. Omykiss3 represents an outgroup in the rooted tree (fig. 9). Numbers associated with nodes are Bayesian posterior probability branch support. Salmonid isoforms still show single divergence (group by isoform, consistent with Dalziel et al. (2014)), but Alewife isoforms are not included in these clades. Alewife α1a arose first, then α1b, and the evolution of the Salmonid isoforms are nested within divergence in Alewives.

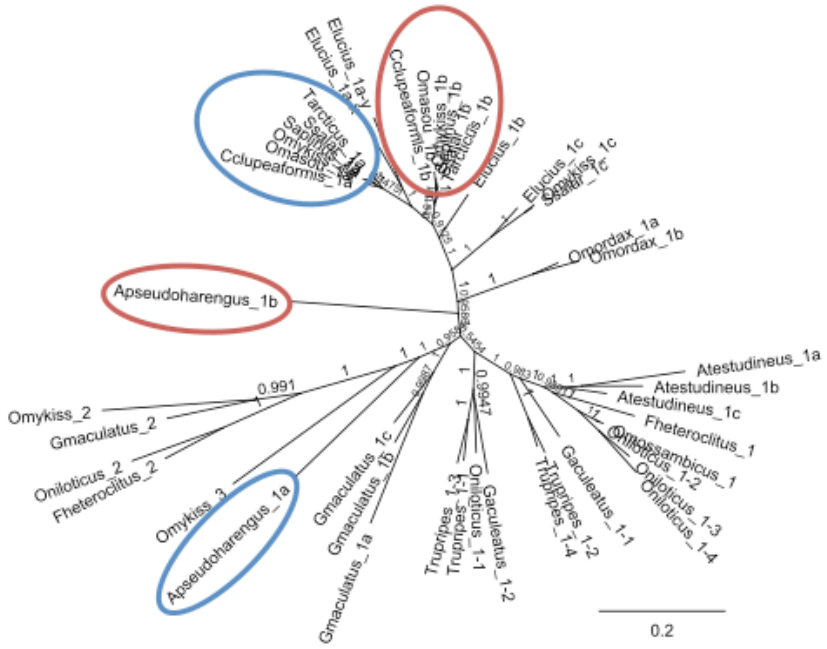

Figure 8: Molecular phylogeny of NKA α1a and α1b in anadromous Alewives and species with previously known sequences. Blue indicates a NKA α1a isoform or clade and red indicates a NKA α1b isoform or clade.

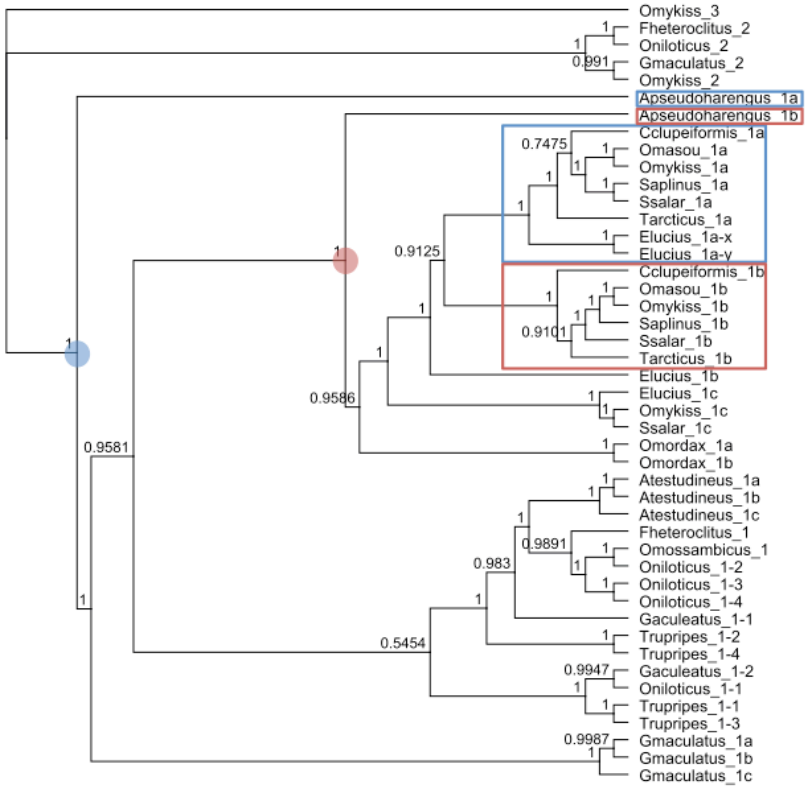

Figure 9: Molecular phylogeny of NKA α1a and α1b in anadromous Alewives and species with previously known sequences. Blue indicates a NKA α1a isoform or clade and red indicates a NKA α1b isoform or clade.

DISCUSSION

The findings of this study represent the first evidence that a member of the family Clupeidae expresses salinity-dependent isoform switching. Naming the isoforms found in this study NKA α1a and α1b is supported by the behavior of expression and sequence similarities. The strong up-regulation of NKA α1a in freshwater and down-regulation in seawater is evident and significant. The up-regulation in seawater and down-regulation in freshwater of α1b is also significant. Further, the early peak in NKA α1b expression (at the 2 or 5 day time period) is characteristic of this isoform and shown in previous studies (Richards et al. 2003, Bystriansky et al. 2006, Urbina et al. 2013). Candidate sequences are also known to be NKA α1 subunits because of nucleotide and protein sequence analyses (conserved regions specific to this family of genes are present). Blastn in NCBI yields mostly isoform specific results. That is to say that all Alewife NKA α1a Blastn hits are NKA α subunit, and most are NKA α1a from other species. The same is true for Alewife NKA α1b Blastn. This evidence supports the claim that the isoforms studied in this project are NKA α1a and α1b.

Statistical analysis supports the alternative hypothesis for population gene expression that landlocked and anadromous Alewives have no statistical difference in NKA α1a and α1b expression at most time points. No significant population effect was found in the mixed model analyses. There are, however, slight trend differences that may indicate expression differences between populations that are discreet. At the 15-day time point, landlocked fish do not express α1a as highly as the anadromous fish. Instead, expression seems to level of at this point, whereas the anadromous population expression continues to increase. This may indicate that landlocked fish are better adapted to the

freshwater environment and have other mechanisms that aid in osmoregulation in low salinity environments. Other mechanisms may include more dilute urine or other transport proteins, and might offset the need for continued up-regulation of α1a.. Though not significant, this finding may be real because it is known from a previous study that landlocked Alewives have significantly reduced performance in seawater compared to anadromous Alewives (Velotta et al, 2014). Also, the trend of α1b expression differs slightly in landlocked compared to anadromous fish. In landlocked fish, α1b peak expression happens at the 5-day time point as opposed to the 2-day maximum seen in anadromous fish. This may also be a consequence of the freshwater specialization of the landlocked population. Landlocked fish never encounter seawater under natural conditions, so they may need more time to up regulate the α1b isoform than anadromous fish that do encounter seawater during their lifetime.

Molecular phylogenetics indicates that patterns of evolution may differ between species of fish. Species that belong to the order Salmoniformes show evidence for evolution in a common ancestor because the isoforms group with like isoforms. The order Salmoniformes underwent a gene duplication event specific to this taxon (Dalziel et al, 2014). Such a duplication event may have allowed the ancestor of these fish to evolve NKA α1a and α1b. Evolution of these isoforms in Salmoniformes may be convergent with Alewives and other unrelated species. The evolution of NKA α1a and α1b in Alewives is unlike what is seen in any other species. The phylogeny indicates that NKA α1a and α1b in Alewives was evolved much earlier than in previously studied species, and that α1a evolved previous to α1b. These findings are plausible because Alewives are

not in the family Salmonidae, and are more distantly related to salmonids than any fishes used in previous studies.

Investigating mechanisms important to euryhalinity is important, because the movement of marine fishes into freshwater is evolutionarily significant. NKA is a key ionoregulator at the gills of fish. This transporter allows fish to maintain a constant internal osmolality against the environment, and without salinity specific forms of this protein, fishes would most likely not have the ability to tolerate different salinity environments. The evolution of these isoforms, therefore, was important to the invasion of freshwater by fishes that lead to much diversification and the origin of tetrapods.

CONCLUSION

Isoform switching is present in many fish taxa, including the relatively basal family Clupeidae. Further work should sequence the Alewife NKA α1a and α1b isoforms, and use the resulting sequence for phylogenetic analysis to investigate the evolutionary origin of these genes. Currently, convergent evolution is favored as the likely mode of evolution for NKA α1a and α1b in different fish lineages, though evolution in a common ancestor is also feasible and may be the most parsimonious solution. Since Alewives are unrelated to any previously studied species, including them in phylogenetic analyses will provide a more diverse sampling of teleost taxa and therefore more strongly supported results.

ACKNOWLEDGMENTS

I would like to thank Dr. Eric Schultz and Jonathan Velotta for helping me get this project up and running, and for providing guidance when needed. I would also like to thank Jeffery Divino, Zachary Skelton, and Mike Smircich for always lending an open ear or a helping hand. For their moral support I thank all the members of the Schultz lab and my friends; you the real MVP's. Thank you to UConn CAGT and Biotechnology facilities for all your help with various molecular techniques. The Michael Alpert and Ariana Napier Honors Enrichment Scholarship, Student Undergraduate Research Fund (SURF) grant generously funded this project, and I am unbelievably grateful for this opportunity

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